

PCT/IL2004/000956

REC'D 23 DEC 2004

WIPO

PCT

PA 1256224

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

December 08, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/512,774

FILING DATE: October 21, 2003

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS



*Trudie Wallace*  
TRUDIE WALLACE

Certifying Officer



**U.S. PATENT AND TRADEMARK OFFICE  
PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT  
under 37 C.F.R. §1.53(b)(2)

Atty. Docket: LEVIN11

INVENTOR(S)/APPLICANT(S)		
LAST NAME	GIVEN NAME	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
LEVIN	Ilan	Mazkeret Batia, Israel
LIEBERMAN	Michal	Rishon Le-Zion, Israel
SEGEV	Orit Amir	Holon, Israel
GILBOA	Nehama	Rison Le-Zion, Israel
LALAZAR	Avraham	Mazkeret Batia, Israel

☐ Additional inventors are being named on separately numbered sheets attached hereto

**TITLE OF THE INVENTION (280 characters max)**

THE TOMATO HOMOLOG OF THE GENE ENCODING UV DAMAGED DNA BINDING PROTEIN 1 (DDB1) IS THE GENE THAT CAUSES THE HIGH PIGMENT-1 MUTANT PHENOTYPE

**CORRESPONDENCE ADDRESS**

Direct all correspondence to the address associated with **Customer Number 001444**, which is presently:

BROWDY AND NEIMARK, P.L.L.C.  
624 Ninth Street, N.W., Suite 300  
Washington, D.C. 20001-5303

**ENCLOSED APPLICATION PARTS (check all that apply)**

<input checked="" type="checkbox"/> Specification	Number of Pages 27	<input type="checkbox"/> Applicant claims small entity status. See 37 C.F.R. §1.27
<input checked="" type="checkbox"/> Drawing(s)	Number of Sheets 7	<input type="checkbox"/> Other (specify) _____

**METHOD OF PAYMENT (check one)**

☒ Credit Card Payment Form PTO-2038 is enclosed to cover the Provisional filing fee of

☒ \$160 large entity      ☐ \$80 small entity

☒ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-4035

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No ☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.

By:

*Sheridan Neimark*  
Registration No.: 20,520

Date: October 21, 2003

SN:gsk

## Introduction

Several photomorphogenic mutants have been described in tomato (*Lycopersicon esculentum*). Among these, mutants carrying the monogenic recessive *high pigment* (*hp-1*, *hp-1<sup>w</sup>*, *hp-2*, and *hp-2<sup>j</sup>*) and *dark green* (*dg*) mutations are characterized by their exaggerated light responsiveness. These mutants display higher anthocyanin levels, shorter hypocotyls, and higher fruit pigmentation than their semi-isogenic normal counterparts (Mochizuki and Kamimura 1984; Wann et al. 1985; Peters et al. 1989; Mustilli et al. 1999; Levin et al. 2003). The higher fruit pigmentation of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, and several flavonoids in the mature ripe red fruit. Because of their effect on fruit color, attributed to enhance lycopene content, *hp* and *dg* mutations have been introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as Lycopene Rich Tomatoes (LRT) (Wann, 1997).

The *hp-1* mutant was originally discovered as a spontaneous mutant in 1917 at the Campbell Soup Company farms (Riverton, NJ) (Reynard, 1956). The *hp-1<sup>w</sup>* mutant appeared among progeny of a plant raised from ethyl methanesulfonate (EMS)-treated seeds of the genotype GT (Peters et al. 1989), the *hp-2* mutant was reported in the Italian San Marzano variety in 1975 (Soressi 1975), the *hp-2<sup>j</sup>* mutant was found among progeny of a T-DNA-transformed plant (cv Moneymaker) (van Tuinen et al. 1997), and the *dg* mutant appeared in trellised planting of the Manapal variety (Konsler 1973). Despite some initial confusion, it is now clear that there are two *HP* genes - *HP-1* and *HP-2* - in the tomato genome, that map to chromosomes 2 and 1, respectively (van Tuinen et al. 1997; Yen et al. 1997). At each of these loci, two of the above mentioned mutant alleles have been initially identified: *hp-1* and *hp-1<sup>w</sup>*, *hp-2* and *hp-2<sup>j</sup>* (Kerckhoffs and Kendrick 1997; van Tuinen et al. 1997).

In a recent study, the *HP-2* gene was cloned and found to encode the tomato homolog of the *Arabidopsis* nuclear protein *DEETIOLATED1* (*DET1*) (Mustilli et al. 1999). In the more phenotypically extreme *hp-2<sup>j</sup>* mutation, a C-to-T transition was found in exon 11, which gave rise to a substitution of a conserved Proline for a Serine residue in the C-terminal region of the DET1 protein. In the *hp-2* mutant, it was shown that an A-to-T transversion directs an alternative splicing of intron 10, leading to a nine-base deletion in exon 11, and resulting in a deletion of the first three amino acids encoded by this exon. The *hp-2* deletion is located within the second putative NLS presumably resulting in mislocalization of the protein. The milder phenotypic display of the *hp-2* mutation might be due to its "leakiness", as about 10% of the normal DET1 protein is produced in the mutant plants (Mustilli et al. 1999).

A comprehensive allele test, carried out by our group, has recently shown that the tomato mutations *dg* and *hp-2<sup>l</sup>* are allelic, in agreement with the exaggerated photomorphogenic de-etiolation response of *dg* mutants grown under modulated light conditions. Sequence analysis of the *DET1* gene in *dg* mutants revealed a single A-to-T base transversion in the second exon of the *DET1* gene in comparison with the normal wild-type sequence. This transversion results in a substitution of a conserved Asparagine<sup>34</sup> by Isoleucine<sup>34</sup> and strongly supports the hypothesis that the tomato *dg* mutation is a novel allele of the tomato homolog of *DET1* gene and, therefore, that it is the *HP-2* locus (Levin et al. 2003).

The gene encoding the *hp-1* and *hp-1<sup>w</sup>* mutant phenotypes has not been identified prior to the present study, although its map location has been clearly characterized (Yen et al. 1997). The findings of the present study suggest that *hp-1* and *hp-1<sup>w</sup>* are alternative alleles at the tomato homolog of the *Arabidopsis* (*A. thaliana*) gene encoding UV DAMAGED DNA BINDING protein 1 (*DDB1*). The *DDB1* protein was recently shown to interact both biochemically and genetically with the *DET1* protein (Schroeder et al. 2002). Our present findings are based on detailed sequence analyses of the tomato *DDB1* gene cloned from both *hp-1* and *hp-1<sup>w</sup>* mutant plants, and a linkage analysis based on the single nucleotide DNA polymorphism (SNP) obtained between *hp-1* cv Ailsa Craig mutant and their corresponding isogenic wild-type plants. In addition, the present study provides the basis for the generation of polymorphic PCR-based DNA markers that can be used for marker-assisted selection of *hp-1* and *hp-1<sup>w</sup>* mutant plants at the seedling stage. This was demonstrated in this study by developing a codominant DNA marker that distinguishes between the *hp-1* and normal alleles utilizing the pyrosequencing genotyping methodology.

## Materials and methods

### Plant material and crosses

Seeds from the normal, open pollinated, tomato (*Lycopersicon esculentum*) cv. Ailsa Craig and a line nearly isogenic and homozygous for the *hp-1* mutation were kindly provided by J.J. Giovannoni, of the Boyce Thompson Institute for Plant Research, Ithaca, NY, USA.

Seeds from cv. Rutgers homozygous for the a *hp-1* mutation (LA3004), as well as seeds from *hp-1<sup>w</sup>/hp-1<sup>w</sup>* mutant plants and their isogenic normal plants in GT background (LA LA4012 and LA4011, respectively), were provided by R.T. Chetelat, of the Tomato Genetics Cooperative, UC Davis, CA, USA. The genotype GT is a tomato breeding line, resistant to mosaic virus, and similar in morphology to cv. Moneymaker, originally obtained from

Deruiterzonen, Bleiswijk, the Netherlands (Koorneef et al. 1990). The *hp-1<sup>w</sup>/hp-1<sup>w</sup>* mutant plants appeared among progeny of a plant raised from EMS-treated seeds of the genotype GT (Peters et al. 1989). Therefore, these plants are highly isogenic to the normal GT genotype. Mutant *hp-1<sup>w</sup>/hp-1<sup>w</sup>* plants show a more extreme phenotype compared to *hp-1/hp-1* plants, and it was clearly shown that *hp-1* and *hp-1<sup>w</sup>* are allelic (Peters et al. 1989).

A processing *hp-1/hp-1* mutant hybrid, LRT89, two *hp-1/hp-1* breeding lines, L525 and L527, and a normal breeding line, N671, were developed by the late R. Frankel, D. Lapushner and I. Levin at the Volcani Center. Seeds from two *hp-1/hp-1* processing hybrids, HA3501 and HA3502, developed by Hazera Genetics Inc., Israel, were provided by Mr. Ezri Peleg. Seeds of the heterozygous *hp-1/+* cultivar, cv. 124, were also provided by Hazera Genetics Inc., Israel. Several normal *+/+* tomato cultivars used in this study, i.e.: Moneymaker, M82, Brigade, VF-36, 189, Manapal, NC8288 and Florida, were from seed stocks available at the Volcani center. DNA was also extracted from single plants of AB427, AB510 and AB747, three *hp-1/hp-1* processing hybrids developed by AB Seeds Inc., Israel.

Normal cv. Ailsa Craig plants were crossed with their nearly isogenic *hp-1* mutant plants to yield F<sub>1</sub> seeds. These F<sub>1</sub> plants were allowed to self pollinate to yield F<sub>2</sub> seeds. A sample of 123 F<sub>2</sub> seedlings was used for the linkage analysis carried out in this study.

#### Genomic DNA extraction and Southern blot hybridization

Throughout this study genomic DNA was extracted from individual plants according to Fulton et al. 1995. To determine the copy number of the *DDB1* gene in the tomato genome, Southern blot hybridization was carried out according to the following procedure: Genomic DNA extracted from both *L. esculentum* (cv. M82) and *L. pennellii* was digested with *Eco*R I, *Eco*R V, *Dra* I, *Hae* III, *Sca* I, and *Mva* I restriction endonucleases. Following electrophoresis in 1.0% agarose gel and Southern transfer, the DNA was hybridized with a P<sup>32</sup> labeled DNA probe containing 1346 bp of the 5' coding sequence of the *DDB1* gene. Southern blot transfer and DNA hybridization were done according to Levin and Smith (1990).

#### Design of PCR primers

Sequence analysis and locus-specific primer design were carried out with the DNAMAN, Sequence Analysis Software version 4.1 (Lynnon BioSoft, Quebec, Canada). All DNA

primers used during the course of this study were purchased from M.B.C Molecular Biology Center Ltd., Ness-Ziona, Israel.

#### PCR reactions

Throughout this study PCR reactions were used for mapping, cloning and amplification of DNA products for direct sequencing and pyrosequencing. For all of these purposes, the amplification reactions (25 µl final volume) were performed with 10 ng template DNA, 25 mM TAPS (pH=9.3 at 25°C), 50 mM KCl, 2mM MgCl<sub>2</sub>, 1mM B-mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 pmoles of each of two primers and 1 unit of thermostable *Taq* DNA polymerase (SuperNova *Taq* polymerase, Madi Ltd., Rishon Le Zion, Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, MA, USA).

For mapping and direct sequencing, initial incubation was at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and polymerization at 72°C for 1-2 min, depending upon the PCR product size. Final polymerization at 72°C was carried out, for 5 min, after completion of the above cycles. The PCR amplification products were visualized by electrophoresis in 1.0% agarose gels and detected by staining with ethidium bromide.

For the PCR amplification preceding the pyrosequencing reaction, initial incubation was at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and polymerization at 72°C for 20 sec. Final polymerization at 72°C was carried out, for 5 min, after completion of the above cycles.

#### Mapping the *DDB1* gene

*DDB1* was mapped by means of *Lycopersicon (L.) pennellii* introgression lines (Eshed et al. 1992). DNA extracted from individual plants of each of the introgression lines, including their original parental lines M82 and *L. pennellii*, were used as templates in PCR reactions. The primers used for these mapping reactions were mTDDB F and mTDDB R (Table 1). These primers were derived from the Institute of Genomic Research (TIGR) database accession TC117372 (<http://www.tigr.org/>) that was found highly homologous to both copies

of the *A. thaliana* *DDB1* gene. To obtain polymorphism between M82 and *L. pennellii*, the PCR products were digested with *Pst* I endonuclease, following the PCR reaction.

#### Cloning and sequencing of the tomato *DDB1* cDNA from *hp-1* and *hp-1<sup>w</sup>* mutant plants

Total RNA was extracted from 25 mg of leaf tissue of individual *hp-1* and *hp-1<sup>w</sup>* mutant seedlings and their nearly isogenic open pollinated wild-type genotypes (Ailsa Craig and GT, respectively). The RNA extraction was carried out using the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, MD, USA). Total RNA was used as the template for first-strand cDNA synthesis using the Superscript pre-amplification system (GibcoBRL Life Technologies, Paisley, UK). The cDNA prepared was used as a template in PCR reactions, to amplify overlapping fragments of the gene encoding the tomato *DDB1*, from in both mutant and normal genetic accessions. The PCR products were then sequenced, either directly or after cloning into pGEM-T Easy Vector using the pGEM-T Easy Vector Systems, according to the manufacturer recommendations (Promega, Madison, WI, USA). After cloning into pGEM-T Easy Vector, four or five independent clones of each of the overlapping amplified fragments were sequenced, based on the vector T7, SP6, and primers complementary to the tomato *DDB1* gene. Whenever direct sequencing was used, at least two PCR products, representing each primer combination complementary to the tomato *DDB1* gene, were sequenced. Sequencing was carried out with an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The 3' region of the tomato *DDB1* gene was directly sequenced by using overlapping fragments amplified with primers complementary to TIGR data base accession TC117372 (<http://www.tigr.org/>) that is highly homologous to both copies of the *A. thaliana* *DDB1* gene. These primers are presented in Table 1.

The 5' region of the *DDB1* gene was initially cloned from a pBluescript® SK(+/-) phagemid cDNA library with the following primers:

T7= 5'-GTAATACGACTCACTATAGGGC-3' and

5'TDDB\_R= 5'-CTGGACTTGAGAATTGAAGCCT-3'

This cDNA library, kindly provided by R. Barg and Y. Salts, of the Volcani Center, Israel, was prepared from young parthenocarpic fruits of 4-6 mm in diameter (ca. 4-8 days post-anthesis) derived from the facultative parthenocarpic determinate line L-179 (*pat-2/pat-2*). This line was described previously (Barg et al. 1990). The library was prepared with the cDNA Synthesis Kit#200400, Zap-cDNA Synthesis Kit#200401, and Zap cDNA Gigapack

III Gold Cloning Kit#200450 of Stratagen Inc., according to the of the manufacturer's instructions.

The 5' region of the tomato *DDB1* gene from *hp1/hp1* and *hp1<sup>w</sup>/hp1<sup>w</sup>* mutant lines and their corresponding nearly isogenic normal lines was directly sequenced using the above primer (5'TDDB\_R) and the primer TDB\_UTR= 5'-ATAGCGGGAAGAGGGAAGATAC-3', that is complementary to the 5' UTR of the tomato *DDB1* gene. Several overlapping primers complementary to the above fragment, such as those used for pyrosequencing genotyping (see below), were used for sequence verification of the 5' coding sequence of the tomato *DDB1* gene.

#### Linkage analysis

The linkage analysis study between the tomato *DDB1* locus and the exaggerated photomorphogenic de-etiolation response characterizing *hp-1* mutant, was carried out using F<sub>2</sub> seeds of a cross between *hp-1* mutant plants and wild-type plants (cv. Ailsa Craig). These seeds were allowed to germinate under a yellow plastic screen that prevented the transmission of light of wavelengths under 500nm (Mochizuki and Kamimura 1984), in an environmentally controlled growth chamber (25°C day/18°C night). These germination and initial growth conditions result in exaggeration of hypocotyl-length differences between the mutant and normal plants (Mochizuki and Kamimura, 1984). The hypocotyl lengths of individual F<sub>2</sub> seedlings were measured 8 days after sowing, and their genotype was determined with the pyrosequencing-based DNA marker developed in the present study.

#### Pyrosequencing genotyping

A pyrosequencing genotyping system, extensively reviewed by Ronaghi 2001, was developed in the present study based on the single nucleotide polymorphism (SNP) discovered in this study between *hp1/hp1* mutant line and its nearly isogenic normal line in cv. Ailsa Craig background. For this purpose a genomic fragment containing the SNP was cloned and sequenced as presented in Fig. 1. The biotin-labeled forward primer for this reaction was 5'-TGTTTTCCAGAGTTACCGGACT-3'; the reverse primer was 5'-TAGCTTGAGCCAATGAAGACAA-3'; and the sequencing primer was 5'-ATGAAGACAAAAGCAT-3'. The amplicon size in this reaction was 106 bp.



The PCR amplification reaction preceding the pyrosequencing reaction was as described above (see PCR reactions). Two pmoles of the sequencing primer were added to the amplification reaction prior to the pyrosequencing analysis. The analysis was carried out using a MegaBASE 1000 instrument (Danyel Biotech, Nes Ziona, Israel). Because the sequencing primer is in reverse orientation, the normal genotype is characterized by T whereas the homozygous mutant *hp-1* genotype is characterized by A at the SNP location, as shown in Fig. 3.

#### Statistical analyses

Analyses of variance (ANOVA) were carried out with the JMP Statistical Discovery software (SAS Institute, Cary, NC, USA). Linkage analysis and LOD score determination were carried out with the QGENE software Version 3.06d (Nelson 1997). Alignment of amino-acid sequences was carried out using the Clustal method (Higgins and Sharp 1988).

#### Results

##### Identification and cloning of the tomato homolog of *DDB1*

The DDB1 protein is a heterodimer consisting of two subunits, DDB1 and DDB2. Unlike rice, chicken, human, mouse, *Drosophila* and *Schizosaccharomyces pombe*, the *A. thaliana* genome harbors two highly homologous copies of the *DDB1* gene (Schroeder et al. 2002; Zolezzi et al. 2002; Fu et al. 2003; Ishibashi et al. 2003): DDB1A, and DDB1B, both 1088 amino-acids in length (Genbank protein accessions NP\_192451 and NP\_193842, respectively). When each of these two protein accessions were used as a query in tblastn analysis against the TIGR database (<http://www.tigr.org/>) containing tomato Expressed Sequence Tags (EST), both revealed two highly homologous sequences: TC117371 (394 bp) and TC117372 (2206 bp). The *A. thaliana* Accession NP\_192451 was found to share 87 and 86% identities with the tomato TC117371 and TC117372 accessions, respectively. Accession NP\_193842, on the other hand, shared 87 and 83% identities with the tomato TC117371 and TC117372 accessions, respectively. Careful sequence analysis, based initially on the longer TIGR accession, TC117372, and later on the single gene that we had cloned from a cDNA library, made it clear to us that the two tomato TIGR accessions, TC117371 and TC117372, were complementary to the same gene sequence. Moreover, Southern-blot transfer and

hybridization of tomato genomic DNA, with the *DDB1* gene sequence as a probe, revealed that indeed the tomato genome contains a single copy of the *DDB1* gene (data not presented).

#### Mapping of the tomato *DDB1*

Partial mapping results, that include the approximate map location of the tomato *DDB1* gene, are presented in Fig. 2. These results indicate that the *DDB1* is located on the tomato chromosome 2, in the introgression line that harbors the *HP-1* gene (Yen et al. 1997).

#### Sequence characterization of the tomato *DDB1* in *hp-1* and *hp-1<sup>w</sup>* mutants

We used several forward and reverse primers (Table 1), complementary to the 3' region of the tomato *DDB1* gene (TIGR accession TC117372), to perform direct sequencing on cDNA prepared from leaves of seedlings from *hp-1* and normal plants in Ailsa Craig background. No polymorphism was obtained between *hp-1* and normal plants in this region. We therefore cloned and thoroughly sequenced the 5' region of the *DDB1* gene in the two genotypes as well. Computerized translation of all sequence results showed that the tomato *DDB1* is a 1090-amino-acid protein. Sequence analysis of the *DDB1* coding sequence from *hp-1* and its nearly isogenic normal genotype revealed a single A<sup>931</sup>-to-T<sup>931</sup> base transversion in the coding sequence of *DDB1* gene of the mutant *hp-1* plants. This transversion resulted in a substitution of a conserved Asparagine<sup>311</sup> to Tyrosine<sup>311</sup> (Fig. 4).

Based on the sequence information obtained in the Ailsa Craig background, we have also sequenced the entire coding region of the *DDB1* gene in *hp-1<sup>w</sup>* mutant plant and its isogenic normal counterpart in GT background. Because *hp-1<sup>w</sup>* is allelic to *hp-1*, a major mutation in the coding sequence of the *DDB1* gene in *hp-1<sup>w</sup>* mutants would strongly support the hypothesis that the tomato *DDB1* gene causes both the *hp-1* and *hp-1<sup>w</sup>* mutant phenotypes. Indeed, a single G<sup>2392</sup>-to-A<sup>2392</sup> transition was observed in the *DDB1* coding sequence in the *hp-1<sup>w</sup>* mutant plant which results in a substitution of a conserved Glutamic-acid<sup>798</sup> to Lysine<sup>798</sup> (Fig. 4).

The complete nucleotide coding sequence and the deduced amino acids sequence of the normal wild-type tomato *DDB1* gene are shown in Fig. 5 and Fig. 6, respectively.

## Genotyping of lines and cultivars

We genotyped 19 lines or cultivars, obtained from various sources, by a combination of direct sequencing and pyrosequencing methods (Fig. 3). Among them were, to the best of our knowledge, a single heterozygous *hp-1/+*, 10 *hp-1/hp-1* and eight normal *+/+* accessions. We found a complete agreement between the SNP identified at the *DDB1* gene and the known genotype of the plants at the *HP-1* locus (results not presented).

## Analysis of the linkage between the *DDB1* locus and the photomorphogenic response

A linkage analysis study was carried out to test the association between the *DDB1* locus and the characteristic hypersensitive-photomorphogenic response displayed by *hp-1* mutant seedlings (i.e., the inhibition of hypocotyl elongation phenotype). For this purpose,  $F_2$  seeds of a cross between determinate *hp-1* mutant plants and wild type plants (cv. Ailsa Craig) were germinated under yellow plastic screen in a controlled growth chamber. Eight days after sowing, the hypocotyls lengths of individual seedlings were recorded, and their *DDB1* locus was genotyped with aid of the pyrosequencing DNA marker, as described above. The results demonstrate a clear association between the *DDB1* locus and hypocotyls length (Table 2). Homozygous recessive *hp-1/hp-1* seedlings displayed a highly significant inhibition of hypocotyl elongation, indicative of a more exaggerated photomorphogenic de-etiolation response, in comparison to the two other two genotypic groups ( $25 < \text{LOD Score} < 26$ ,  $R^2 = 62.8\%$ ). These results confirm that the mutation identified in the *DDB1* locus of *hp-1* mutant plants is associated with one of its main characteristic phenotypes, i.e., inhibited hypocotyl elongation in the seedlings. Interestingly, a slight partially dominant effect for the *hp-1* allele was obtained in this study. This effect can be noted from the statistically significant differences obtained between the *+/+* and *hp-1/+* group means (Table 2).

## Discussion

Plants respond to light by modulating their developmental processes, in an array of interactions that are commonly and collectively referred to as photomorphogenesis. Photomorphogenic mutants have proven to be an excellent tool in studying the complex interactions between light and plant development. In general, these mutants may be classified either as defective in photoreceptors, or altered in some elements of the light signal

transduction chain (Chory 1993). Photomorphogenesis has been intensively studied in the model species *A. thaliana*. It is however difficult to carry out the appropriate biochemical studies or to test hypotheses related to the interaction between light and fruit quality, on its small, dry fruits. The tomato, on the other hand, is characterized by a fleshy fruit that accumulates phytochemicals. The tomato homolog of the photomorphogenic *DEETIOLATED1* (*DET1*) gene has recently been characterized as the gene carrying three light-sensitive mutations: *high pigment-2* (*hp-2*), *high pigment-2'* (*hp-2'*) and, recently, *dark green* (*dg*), but these have been only partially characterized and studied (Mustilli et al. 1999; Levin et al. 2003). The exaggerated light responsiveness that typifies these mutants leads to significantly higher anthocyanin levels in their developing seedlings than those of their semi-isogenic normal counterparts. These mutants are also characterized by higher fruit and foliage pigmentation. The high fruit pigmentation of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, in the mature ripe-red fruit. Due to their effect on fruit color, attributed mainly by higher lycopene content, *hp* and *dg* mutations have been introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as Lycopene Rich Tomatoes (LRT) (Levin et al 2003).

Interestingly, the *hp* and *dg* mutations are also characterized by overproduction of several flavonoids and of vitamin C in the mature fruit (Mochizuki and Kamimura 1984; Yen et al. 1997). This overproduction is associated with increased plastid biogenesis, and therefore overproduction of other metabolites may be expected in such mutants. As such, these mutations, demonstrate a possible conceptual link between overproduction of primary and secondary metabolites and photomorphogenesis. Tomato plants carrying the *high pigment* (*hp*) and *dark green* (*dg*) mutations therefore represent a unique, non-GMO platform for the production of health-promoting functional tomatoes.

Historically, the nomenclature of the *hp* and *dg* mutants has been somewhat confusing as was recently summarized (Jones et al. 2001). Also, for years it has been speculated that *hp* and *dg* represent mutations in structural genes of the carotenoid biosynthesis pathway (Stevens and Rick 1986). Although the photomorphogenic nature of these mutants could have been elucidated several years ago (Mochizuki and Kamimura 1984; Peters et al. 1989; Peters et al. 1992), its possible link to carotenogenesis was largely ignored. An elegant study, published recently, initiated a conceptual link between carotenogenesis control and photomorphogenesis, by demonstrating that the phenotypes of the tomato *hp-2* and *hp-2'* mutants are caused by mutations in the tomato homolog of *DET1* (Mustilli et al. 1999). We have recently identified *dg* as an additional mutation at the *DET1* locus (Levin et al. 2003).

The similarities in the pleiotropic phenotypes expressed in seedlings, mature plants and developing and rip-red fruits between the *dg* mutation and all known *hp* mutations were recognized (Mochizuki and Kamimura, 1984), but the molecular link was incomplete prior to our present study. These phenotypic similarities led us to map candidate photomorphogenic genes, in an attempt to identify the gene that encodes the *hp-1* mutant phenotype (data not shown). However, none of those genes was found to map in close association with the *HP-1* locus. An epitope-tagging approach, recently applied in *A. thaliana*, showed that the DET1 protein is localized to the nucleus and forms an approximately 350 kDa complex, which is required for full DET1 activity (Schroeder et al. 2002). A 120 kDa protein that is the plant homolog of DDB1, a protein implicated in the human disease *Xeroderma pigmentosum* was affinity purified from that complex.

Comprehensive molecular and genetic tests, carried out in the course of the present study, demonstrated that *hp-1* mutant plants are characterized by a substitution of Asparagine<sup>311</sup> to Tyrosine<sup>311</sup> in the DDB1 protein (Fig. 4). We also demonstrated that in the phenotypically extreme *hp-1<sup>w</sup>* mutation, previously shown to be allelic to *hp-1* (Peters et al. 1989), an acidic Glutamic-acid<sup>798</sup> is substituted by a basic Lysine<sup>798</sup> in the same protein (Fig. 4). Both, Asparagine<sup>311</sup> and Glutamic-acid<sup>798</sup> are highly conserved residues of the DDB1 protein in both plant and animal species (Fig. 4). In a linkage analysis we further showed that the characteristic photomorphogenic phenotype of early seedling hypocotyl growth of *hp-1* mutants is very highly associated with the mutation observed at the *DDB1* locus of *hp-1/hp-1* genotypes (Table 2). These results therefore suggest that *hp-1* and *hp-1<sup>w</sup>* mutant phenotypes are caused by mutations in the tomato homolog of the *A. thaliana* *DDB1* gene.

DDB is a heterodimer, comprised of two subunits: DDB1 and DDB2. DDB is an important factor involved in DNA repair and cell cycle regulation and it has high affinity for a variety of DNA lesions including UV-photoproducts (Fu et al. 2003; Ishibashi et al. 2003). DDB1 was originally identified as a component of UV-Damaged DNA Binding activity absent in *Xeroderma pigmentosum* complementation group E patients (Chu and Chang, 1988; Keeney et al. 1993). The molecular lesions in these patients, however, were later mapped to DDB1's binding partner, DDB2 (Nichols et al. 1996; Hwang et al. 1998). Both DDB1 and DDB2 were found to interact with either histone acetyltransferase (HAT) proteins or HAT complexes (Brand et al. 2001; Datta et al. 2001; Martinez et al. 2001). Recently it was also shown that the tomato DET1 interacts with histones, specifically the nonacetylated tail of H2B, both in vitro and in vivo (Benvenuto et al. 2002). These protein interactions, as well as the involvement of DET1 in the expression of many light-regulated genes have led to the

suggestion that DET1 and DDB1 regulate gene expression via an interaction with chromatin (Schroeder et al. 2002).

In *A. thaliana* and tomato, *det1* mutants exhibit many characteristics of light-stressed plants. These characteristics were thoroughly characterized in *A. thaliana*, following a detailed microarray analysis showing that the expression of many genes associated with various stress responses, including light stress, are misregulated in *det1* mutant plants (Hu et al. 2002; Schroeder et al. 2002). The interaction of DET1 with DDB1, a protein that protects humans from damaging UV radiation, as well as our results present finding that the light-sensitive *hp-1* and *hp-1<sup>w</sup>* mutations are in fact lesions in the gene that encode DDB1 protein, further reinforce the hypothesis that the roles of these proteins may have evolved from a common mechanism for managing light stress (Schroeder et al. 2002).

Interestingly, in itself, a null mutation in the *A. thaliana* *DDB1A* results in no obvious phenotype on its own, yet it enhances the phenotype of a weak *det1* allele. In *A. thaliana*, however, there are two highly homologous copies of the *DDB1* exist that share 89 and 87% identities at the amino-acids and nucleotide level, respectively. Therefore, a mutation in *DDB1A* may be compensated for by the other copy, i.e., *DDB1B*. Southern blot hybridization, carried out in our present study (data not shown), indicated that *DDB1* is a single-copy gene in the tomato genome, as has been found also in rice, chicken, humans, mouse, *Drosophila* and *Schizosaccharomyces pombe* (Schroeder et al. 2002; Zolezzi et al. 2002; Fu et al. 2003; Ishibashi et al. 2003). A mutation in such a single-copy gene may, generate a much greater phenotypic effect than that in *A. thaliana*, as can be observed in *hp-1* and *hp-1<sup>w</sup>* mutants of tomatoes. Differences were also observed between the tomato and *A. thaliana* *det1* mutants in spite of the significantly high DET1 protein-homology that exist between them. Whereas the *Arabidopsis* mutations elicit their phenotype under total darkness, the tomato mutations require a limited illumination at the phytochrome functional spectrum (Red/Far Red), in order to display their phenotypic expression (Peters et al. 1992). Indeed, in full accordance with the light requirement specificity, the tomato *hp* mutations do need active phytochromes for functional display whereas the *Arabidopsis* mutations do not (Pepper et al. 1994; Mustilli et al. 1999).

The apparent effects of mutations at the tomato *DET1* locus, and – in light of the findings of the present study – at the DET1 protein complex, including the DDB1 protein, on the increased production of health-promoting carotenoids, flavonoids and vitamins have already been documented (Mochizuki and Kamimura 1984; Wann et al. 1985; Yen et al. 1997; Levin et al. 2003). Such effects suggest that genes active in the light signal transduction cascade

may be important candidate genes for association with quantitative trait loci that affect such important metabolite levels in the tomato fruit. *DET1* and *DDB1* homologs were also found in a wide range of species distantly related to the tomato, such as humans and *Drosophila*. This suggests that effects of light-responsive genes on the production of health-promoting compounds in other plant species should not be ignored. From such a practical point of view, the present study has identified a polymorphic DNA marker that can be used as a marker-assisted selection tool for the identification of the *hp-1* and *hp-1<sup>w</sup>* mutant alleles and for combining these alleles with alleles that have already been mapped to the unlinked *HP-2* gene (Mustilli et al. 1999; Levin et al. 2003). Such a tool could aid the introgression of the *hp-1* or *hp-1<sup>w</sup>* mutation into various genetic backgrounds for the purpose of improving the fruit quality and nutritional value of tomatoes.

Further non-limiting examples (both working and theoretical) that illustrate and describe various practical embodiments of the present invention are given in the following section. These embodiments are described for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

#### Example 1

##### Diagnostic tool for identifying the *hp-1* and *hp-1<sup>w</sup>* mutations

A pyrosequencing DNA marker system, extensively reviewed by Ronaghi 2001, for use as a molecular diagnostic tool for identifying *hp-1* mutant plants based on the sequence results (Fig. 1) was developed. This DNA marker is based on the single nucleotide polymorphism (SNP) discovered in this study between *hp1/hp1* mutant line and its nearly isogenic normal line in cv. Ailsa Craig background. For this purpose a genomic fragment containing the SNP was cloned and sequenced. The sequence of this genomic fragment is presented in Fig. 1. The biotin labeled forward primer for this reaction was: 5'-TGT'TTTCCAGAGTTACCGGACT-3'; the reverse primer was: 5'-TAGCTTGAGCCAATGAAGACAA-3'; and the sequencing primer was 5'-ATGAAGACAAAAGCAT-3'. The amplicon size in this reaction was 106 bp.

The PCR amplification reaction preceding the pyrosequencing reaction was as described (see PCR reactions above). Two pmoles of the sequencing primer were added to the amplification reaction prior to the pyrosequencing analysis. The analysis was carried out using a MegaBASE 1000 instrument by Danyel Biotech, Nes Ziona, Israel. Because the

sequencing primer is in reverse orientation, the normal genotype is characterized by T whereas the homozygous mutant *hp-1* genotype is characterized by A at the SNP location as presented in fig. 3.

Using the pyrosequencing methodology and the primers described above a clear polymorphism between *hp-1* and wild-type plants was seen as demonstrated in Fig. 3. In the case of the homozygous *hp-1* mutant plants, a single peak representing Adenine (A) at the SNP location was observed, while in wild-type plants, a single peak representing Thymine (T) at the SNP location was observed (Fig. 3). As expected, plants heterozygous for the *hp-1* mutation yielded two peaks, representing both A and T nucleotides (Fig. 3).

A similar pyrosequencing based marker system can be established based on the SNP observed in *hp-1<sup>w</sup>* mutant plants.

### Example 2

#### Linkage analysis between the *DDB1* locus and the photomorphogenic response

A linkage analysis study between the tomato *DDB1* locus and the exaggerated photomorphogenic de-etiolation response characterizing *hp-1* mutant, was carried out using  $F_2$  seeds of a cross between *hp-1* mutant plants and wild type plants (cv. Ailsa Craig). These seeds were allowed to germinate under yellow plastic screen, omitting transmittance of light spectra under 500nm (Mochizuki and Kamimura, 1984), in an environmentally controlled growth chamber (25°C day/18°C night). These germination and initial growth conditions result in exaggeration of hypocotyls-length differences between the mutant and normal plants (Mochizuki and Kamimura, 1984). Hypocotyl-length of individual  $F_2$  seedlings was measured 8 days after sowing and their genotype determined using the pyrosequencing based DNA marker developed in this study. The results demonstrate a clear association between the *DDB1* locus and hypocotyl-length (Table 2). Homozygous recessive *hp-1/hp-1* seedlings displayed a highly significant inhibition of hypocotyl elongation, indicative of a more exaggerated photomorphogenic de-etiolation response, in comparison to the two other genotypic groups ( $25 < \text{LOD Score} < 26$ ,  $R^2 = 62.8\%$ ). These results confirm that the mutation identified in the *DDB1* locus of *hp-1* mutant plants is associated with one of its main characteristic phenotypes, i.e. inhibited hypocotyl elongation of seedlings. Interestingly, a slight partial-dominant effect was obtained in this study for the *hp-1* allele. This effect can be



noted from the statistical significant differences obtained between the  $+/+$  and  $hp-1/+$  group means (Table 2).

### Example 3

#### Incorporation of two genetically unlinked lycopene enhancing mutations in a single tomato hybrid: *Experimental Approach*

A common practice among breeders is to combine or incorporate two or more mutations positively affecting the same trait. Such procedure can be verified by laborious and time consuming test crosses. The diagnostic tool produced herein can facilitate the incorporation of two light hypersensitive lycopene-enhancing mutations in a single plant or breeding line.

Several mutations in tomato positively affect lycopene content in the mature tomato fruit. Of these, at least 5 show a significant hypersensitive light response. These include:

1. *High pigment-1 (hp-1)*
2. *High pigment-1<sup>w</sup> (hp-1<sup>w</sup>)*
3. *High pigment-2 (hp-2)*
4. *High pigment-2<sup>j</sup> (hp-2<sup>j</sup>)*
5. *Dark green (dg)*

The  $hp-1$  and  $hp-1^w$  mutations map to the  $HP-1$  locus on the tomato chromosome 2 (Yen et al. 1997 and in accordance with the present invention). The  $hp-2$ ,  $hp-2^j$  and  $dg$  mutations map to the  $HP-2$  locus on the tomato chromosome 1 (Mustilli et al. 1999; Levin et al. 2003). Incorporation of lycopene enhancing  $hp-2$ ,  $hp-2^j$  or  $dg$  at the  $HP-2$  locus and either one of the two mutations that map to the  $HP-1$  locus ( $hp-1$  and  $hp-1^w$ ) can be more efficiently achieved through the following procedure (illustrated for the  $dg$  and  $hp-1$  mutations):

1. Cross homozygous *dg* with homozygous *hp-1* mutants to generate double heterozygous F<sub>1</sub> plants:

$$dg/dg \ +/+ \times \ +/+ \ hp-1/hp-1$$

$$\downarrow$$

$$dg/+ \ hp-1/+$$

2. Self-cross the F<sub>1</sub> double heterozygous plants to generate F<sub>2</sub> seeds. These F<sub>2</sub> seeds will segregate into 9 genotypes: *dg/dg hp-1/hp-1*, *dg/dg hp-1/+*, *dg/dg +/+*, *dg/+ hp-1/hp-1*, *dg/+ hp-1/+*, *dg/+ +/+*, *+/+ hp-1/hp-1*, *+/+ hp-1/+*, *+/+ +/+*.

Using the pyrosequencing marker system developed in this application for the *hp-1* mutation and the marker developed by us for the *dg* mutation (PCT XXXXXXXX), the double homozygous plants *dg/dg hp-1/hp-1* can be easily identified and self-crossed to yield a breeding line homozygous for the two mutations.

#### Example 4

##### Incorporation of two genetically unlinked lycopene enhancing mutations in a single tomato hybrid significantly increases lycopene yield: Working Example

Two semi-isogenic hybrids, one homozygous for the *hp-1* mutation, *hp-1/hp-1*, and the other for the *dg* mutation, *dg/dg*, were crossed hybridized to yield F<sub>1</sub> plants (*hp-1/+ dg/+*). These F<sub>1</sub> plants were self-hybridized to yield F<sub>2</sub> seedlings. These F<sub>2</sub> seedlings were genotyped and self-hybridized to yield double mutant plants (*hp-1/hp-1 dg/dg*), as outlined in Example 3. Two horticulturally acceptable plants were selected and allowed to self hybridize to yield two F<sub>4</sub> lines. These F<sub>4</sub> lines were cross hybridized to yield a double mutant hybrid. This hybrid was tested, together with the semi-isogenic single mutant hybrids used in the initial cross (see above), in 4 locations in northern Israel during the spring season under open field conditions. Results presented in Table 3 show that, unexpectedly, the lycopene yield of the double mutant hybrid is statistically higher compared to its isogenic single mutant hybrids. The increase in lycopene yield of the

double mutant hybrid was 19 and 61% compared to the lycopene yield of the *dg/dg* and *hp-1/hp-1* single mutant hybrids, respectively.

#### Example 5

##### Use of the diagnostic tool for post control analysis of parental lines and hybrid seeds.

Seed companies often use a battery of molecular markers for post- or quality- control of parental seed stocks and hybrid-seeds. Several commercial lycopene-rich tomato cultivars carry the *hp-1* and *hp-1<sup>w</sup>* mutation either at a homozygous or heterozygous state. Up until now, detection of the *hp-1* and *hp-1<sup>w</sup>* traits within a particular stock could only be performed by the lengthy procedure of germinating samples of the seeds, and performing complicated phenotypic analyses on the parental cultivars and subsequent generations.

The diagnostic tool demonstrated in this study (see Example 1, hereinabove) can be used to positively detect the *hp-1* and *hp-1<sup>w</sup>* alleles in such cultivars and their parental lines, and thus enable post-production quality control to be carried out over a time scale of 1-2 days instead of weeks or months.

#### Example 6

##### Mapping of other functionally active mutations in the *DDB1* gene

Seeds extracted from normal plants can be mutagenized with ethyl methanesulfonate (EMS) or other approaches according to known protocols to yield photomorphogenic mutants (Koornneef et al. 1990). These mutants can be selected for under modulated light conditions, such as yellow plastic screen. Photomorphogenic mutants obtained can be screened for unique expression patterns of health-promoting metabolites. These mutants can be further characterized by allele tests against *hp-1* and/or *hp-1<sup>w</sup>* and some of them may be characterized as allelic to these mutations. Thus, mutagenized plants allelic to *hp-1* and/or *hp-1<sup>w</sup>* can be discovered that also bear unique metabolite profiles. Sequence analysis of the *DDB1* gene in these plants should reveal the exact genetic modifications that underline such unique metabolic architectures. These genetic modifications should enable the design of specific molecular markers, similar to those outlined hereinabove, for marker

assisted selection. Also, mapping of such lesions may unravel regions within the *DDB1* gene as targets for efficient genetic manipulation to obtain plants with unique metabolite profiles in the tomato fruit.

#### Example 7

#### Over-expression of modified *DDB1* genes obtained from *hp-1* and *hp-1<sup>m</sup>* mutant plants to obtain over-production of health promoting metabolites in other species of fruits and vegetables

The *DDB1* gene is highly conserved across many evolutionary distant species (Schroeder et al. 2002). Also, its link to overproduction of health-promoting metabolites has been outlined hereinabove. These results suggest that effects of the *DDB1* gene on the production of health promoting compounds should not be ignored in other plant species as well. From such practical point of view, modified *DDB1* genes may be cloned from *hp-1* and/or *hp-1<sup>m</sup>* mutant tomato plants and over-expressed in other plant species to obtain increases in the production of functional metabolites in fruits and vegetables.

## References

- Barg R, Meir E, Lapushner D, Frankel R, Salts Y (1990) Differential regulation of fruit specific 62 kDa protein in developing parthenocarpic (pat-2/pat-2) and seeded tomato fruits. *Physiol Plant* 80:417-424
- Benvenuto G, Formiggini F, Laflamme P, Malakhov M, Bowler C. (2002) The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. *Curr Biol* 12:1529-1534
- Brand M, Moggs JG, Oulad-Abdelghani M, Lejeune F, Dilworth FJ, Stevenin J, Almouzni G, Tora L (2001) UV-damaged DNA-binding protein in the TFIIIC complex links DNA damage recognition to nucleosome acetylation. *EMBO J.* 20:3187-3196
- Chory J (1993) Out of darkness: mutants reveal pathways controlling light-regulated development in plants. *Trends Genet* 9:167-172
- Chu G, Chang E. (1988) *Xeroderma pigmentosum* group E cells lack a nuclear factor that binds to damaged DNA. *Science* 242:564-567
- Datta A, Bagchi S, Nag A, Shiyanov P, Adami GR, Yoon T, Raychaudhuri P (2001) The p48 subunit of the damaged-DNA binding protein DDB associates with the CBP/p300 family of histone acetyltransferase. *Mutat Res* 486:89-97
- Eshed Y, Abu-Abied M, Saranga Y, Zamir D (1992) *Lycopersicon esculentum* lines containing small overlapping introgressions from *L. pennellii*. *Theor Appl Genet* 83:1027-1034
- Fu D, Wakasugi M, Ishigaki Y, Nikaido O, Matsunaga T (2003) cDNA cloning of the chicken DDB1 gene encoding the p127 subunit of damaged DNA-binding protein. *Genes Genet Syst* 2003 78:169-77
- Fulton TM, Chunwongse J, Tansley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Mol Biol Rep* 13:207-209
- Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237-244.
- Hu J, Aguirre M, Peto C, Alonso J, Ecker J, Chory J (2002) A role for peroxisomes in photomorphogenesis and development of *Arabidopsis*. *Science* 297:405-409
- Hwang BJ, Toering S, Francke U, Chu G (1998) p48 Activates a UV-damaged-DNA binding factor and is defective in *Xeroderma pigmentosum* group E cells that lack binding activity. *Mol Cell Biol* 18:4391-4399
- Ishibashi T, Kimura S, Yamamoto T, Furukawa T, Takata K, Uchiyama Y, Hashimoto J, Sakaguchi K (2003) Rice UV-damaged DNA binding protein homologues are most abundant in proliferating tissues. *Gene* 308:79-87

- Jones CM, Myers JR, Chetelat RT (2001) Allele test of high pigment genotypes using root anthocyanin expression. TGC Rep 51:23-26
- Keeney S, Chang GJ, Linn S (1993) Characterization of a human DNA damage binding protein implicated in *Xeroderma pigmentosum* E. J Biol Chem 268:21293-21300.
- Kerckhoffs LHJ, Kendrick RE (1997) Photocontrol of antocyanin biosynthesis in tomato. J Plant Res 110:141-149
- Konsler TR (1973) Three mutants appearing in 'Manapal' tomato. HortSci 8:331-333
- Koornneef M, Bosma TDG, Hanhart CJ, van der Veen JH, Zeevaart JAD (1990) The isolation and characterization of gibberellin-deficient mutant in tomato. Theor Appl Genet 80:852-857
- Kramer CY (1956) Extension of multiple range tests to group means with unequal number of replications. Biometrics 12:309-310
- Levin I, Frankel P, Gilboa N, Tanny S, Lalazar A (2003) The tomato dark green mutation is a novel allele of the tomato homolog of the DEETIOLATED1 gene. Theor Appl Genet 106:454-460
- Levin I, Smith EJ (1990) Molecular analysis of endogenous virus ev21-slow feathering complex of chickens. 1. Cloning of proviral-cell junction fragment and unoccupied integration site. Poult Sci 69:2017-2026
- Martinez E, Palhan VB, Tjernberg A, Lyman ES, Gamper AM, Kundu TK, Chait BT, Roeder RG (2001) Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo. Mol Cell Biol. 21:6782-6795
- Mochizuki T, Kamimura S (1984) Inheritance of vitamin C content and its relation to other characters in crosses between *hp* and *og* varieties of tomatoes. Eucarpia Tomato Working Group. Synopsis IX meeting 22-24 May, Wageningen, the Netherlands, pp 8-13
- Mustilli AC, Fenzi F, Ciliento R, Alfano F Bowler C (1999) Phenotype of the tomato *high pigment-2* is caused by a mutation in the tomato homolog of *DEETIOLATED1*. Plant Cell 11:145-157
- Nelson JC (1997) QGENE: software for marker-based genomic analysis and breeding. Mol Breed 3:229-235
- Nichols AF, Ong P, Linn S (1996) Mutations specific to the *Xeroderma pigmentosum* group E Ddb- phenotype. J Biol Chem. 271:24317-24320
- Pepper A, Delaney T, Washburn T, Poole D, Chory J (1994) *DET1*, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear-localized protein. Cell 78:109-116

Peters JL, van Tuinen A, Adamse P, Kendrick RE, Koornneef M (1989) High pigment mutants of tomato exhibit high sensitivity for phytochrome action. *J Plant Physiol* 134:661-666

Peters JL, Schreuder MEL, Verduin SJW, Kendrick RE (1992) Physiological characterization of high-pigment mutant of tomato. *Photochem Photobiol* 56:75-82

Reynard GB (1956) Origin of Webb Special (Black Queen) in tomato. *Rep Tomato Genet Coop* 40: 44-64

Ronaghi M (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Res* 11:3-11

Schroeder DF, Gahrtz M, Maxwell BE, Cook RK, Kan JM, Alonso JM, Ecker JR, Chory J (2002) De-etiolated 1 and damaged DNA binding protein 1 interact to regulate Arabidopsis photomorphogenesis. *Curr Biol* 12:1462-1472

Soressi GP (1975) New spontaneous or chemically-induced fruit ripening tomato mutants. *Rep. Tomato Genet Coop* 25:21-22

Stevens MA, Rick CM (1986) Genetics and breeding. In: Atherton, JG, and Rudich J (eds) *The tomato crop*. Chapman and Hall, New York, pp 87-90

Table 1. Forward (F) and reverse (R) primers, complementary to TIGR database accession TC117372, used to sequence the 3' region of the tomato *DDB1* gene.

Primer name	Primer sequence
5TDDB F	5'-ACGACCTATCGTGGACTTCTGT-3'
5TDDB R	5'-CTGGACTTGAGAATTGAAGCCT-3'
ln5TDDB F	5'-GAGCCTATAAGGATGGATCAC-3'
ATDDB F	5'-CAGCAGTTGGAATGTGGACAG-3'
mTDDB F	5'-GCAATCGCTAAAGAAGGTGAGT-3'
mTDDB R	5'-GCATTATAGTCTCTGGCTCGCT-3'
inmTDDB F	5'-GGACATTTGCTCTATGCAGT-3'
inmTDDB R	5'-AGGCATTTAGAGAGTAGACAGC-3'
TDDB F	5'-TTTGGAGAAGCTGCAGACAA-3'
TDDB R	5'-CACAACTCACAGAAGAAGAAG-3'
ln3TDDB R	5'-CCACTCTCTTCATTAGTTCCTC-3'



Table 2. Linkage analysis between the tomato *DDB1* locus and the photomorphogenic response displayed by *hp-1* mutant seedlings. Seedlings were grown under a yellow plastic screen for 8 days after sowing. Different superscript letters indicate statistically significant differences between means ( $P < 0.05$ ) according to the Tukey-Kramer HSD test (Kramer 1956).

Genotype	N	Hypocotyl length $\pm$ S.E. (cm)	LOD score	R <sup>2</sup>
+/+	35	9.6 <sup>A</sup> $\pm$ 0.2	25 < LOD < 26	62.8%
<i>hp-1</i> /+	68	8.7 <sup>B</sup> $\pm$ 0.2		
<i>hp-1</i> / <i>hp-1</i>	20	4.2 <sup>C</sup> $\pm$ 0.2		

**Table 3.** Lycopene yield of single and double mutant hybrid cultivars carrying hypersensitive lycopene-enhancing mutations. Different superscript letters represent statistically significant differences between means ( $P < 0.05$ ) based on Tukey-Kramer HSD test (Kramer, 1956).

Cultivar genotype	Lycopene yield (gr/dunam*)
<i>+/+ hp-1/hp-1</i>	1136 <sup>C</sup>
<i>dg/dg +/+</i>	1538 <sup>B</sup>
<i>dg/dg hp-1/hp-1</i>	1824 <sup>A</sup>

\* 1 dunam= 1000 square meters

## Figure Legends

Fig. 1. The genomic fragment used to design pyrosequencing primers for the *hp-1* mutation (the single nucleotide polymorphism is in underlined large bold letters, the forward and the reverse primers are underlined and the sequencing primer is in italic).

Fig. 2. Partial mapping results of the tomato *DDB1* gene (map of the tomato chromosome 2, showing the location of the *HP-1* gene (*hp*) was adopted from Yen et al. (1997)).

Fig. 3. Typical pyrosequencing genotyping results for the *hp-1* mutation at the *DDB1* locus (Note that because the reverse orientation of the sequencing primer, the mutant genotype is characterized by A and the normal genotype by T).

Fig. 4. Partial ClustalW protein Alignment of DDB1 showing the location of the *hp-1* (a) and *hp-1<sup>w</sup>* (b) amino-acid substitutions [presented are *Arabidopsis* DDB1A (At\_DDB1A=NP\_192451), *Arabidopsis* DDB1B (At\_DDB1B=NP\_193842), tomato cv. Ailsa Craig (Le), rice (Os=BAB20761), human (Hs=DDB1\_Human), *Drosophilla* (Dm=XP\_081186), chicken (Gg=BAC56999), and *S. pombe* (Sp= NP\_593580)]. Identical residues are black shaded whereas similar residues are gray shaded.

Fig. 5. Complete nucleotide coding sequence of the normal wild-type tomato *DDB1* gene (the start, ATG, and the termination, TAG, codons are underlined. Location of A<sup>931</sup> and G<sup>2392</sup>, whose transversion and transition leads to the *hp-1* and *hp-1<sup>w</sup>* phenotypes, respectively, are in large bold letters).

Fig. 6. Complete amino-acids sequence of the normal wild-type tomato *DDB1* gene (Asparagine<sup>311</sup> and Glutamic-acid<sup>798</sup> whose substitution leads to the *hp-1* and *hp-1<sup>w</sup>* phenotypes, respectively, are in large bold letters).

The tomato homolog of the gene encoding UV DAMAGED DNA BINDING Protein 1 (*DDB1*) is the gene that causes the *high pigment-1* mutant phenotype

#### Abstract

A tomato EST sequence highly homologous to the human and *Arabidopsis (A.) thaliana* UV DAMAGED DNA BINDING Protein 1 (*DDB1*) was mapped to the centromeric region of the tomato chromosome 2. The entire coding region of the *DDB1* gene was sequence characterized in an *hp-1* mutant and its nearly-isogenic normal plant in cv. Ailsa Craig background, and also in an *hp-1<sup>w</sup>* mutant and its isogenic normal plant in the GT breeding-line background. Sequence analysis revealed a single A<sup>931</sup>-to-T<sup>931</sup> base transversion in the coding sequence of *DDB1* gene in *hp-1* mutant plants. This transversion results in a substitution of a conserved Asparagine<sup>311</sup> to Tyrosine<sup>311</sup>. In the GT background, on the other hand, a single G<sup>2392</sup>-to-A<sup>2392</sup> transition was observed, resulting in a substitution of a conserved Glutamic-acid<sup>798</sup> to Lysine<sup>798</sup>. The single nucleotide polymorphism that differentiates *hp-1* mutant and normal plants in cv. Ailsa Craig background was used to design a pyrosequencing genotyping system. Analysis of a resource F<sub>2</sub> population segregating for the *hp-1* mutation revealed a very strong linkage association between the *DDB1* locus and the photomorphogenic response of the seedlings, measured as hypocotyl length (25<LOD Score<26, R<sup>2</sup>= 62.8%). These results underline *DDB1* as the gene encoding the *hp-1* and *hp-1<sup>w</sup>* mutant phenotypes.

#### Brief Description of the Present Invention

It has now been found that the mutations responsible for both the *hp-1* and *hp-1<sup>w</sup>* mutant phenotypes are located within the tomato homolog of the human and *Arabidopsis thaliana* UV DAMAGED DNA BINDING Protein 1 (*DDB1*) gene.

The present invention is thus primarily directed to isolated nucleotide sequences responsible for the tomato *hp-1* and *hp-1<sup>w</sup>* phenotypes, wherein said sequence comprises an altered tomato *DDB1* gene sequence or fragment or homolog thereof. In the case of the *hp-1* mutation, the alteration in said sequence or fragment or homolog comprises a single A<sup>931</sup>-to-

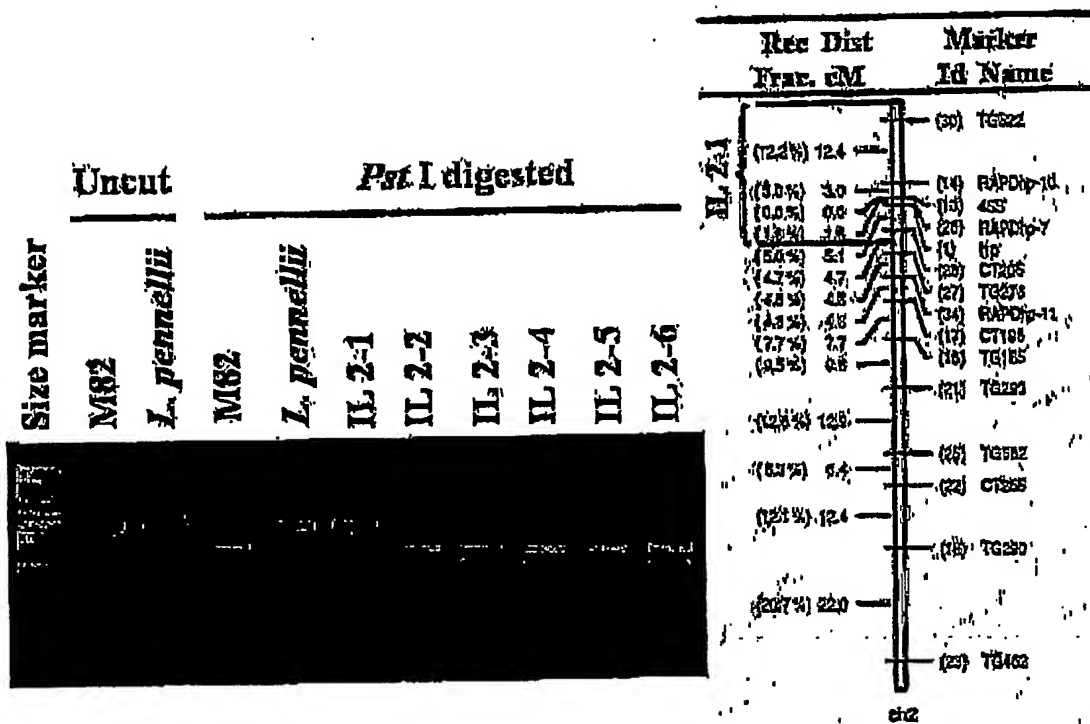
T<sup>931</sup> base transversion in the tomato homolog of the *DDB1* coding sequence. In the case of the *hp-1<sup>w</sup>* mutation, the alteration in said sequence or fragment or homolog comprises a single G<sup>2392</sup>-to-A<sup>2392</sup> transversion in the tomato homolog of the *DDB1* coding sequence.

The present invention is particularly directed to the use of the abovementioned isolated mutant nucleotide sequences as a molecular diagnostic tool. Said tool may be used to aid the introgression of the *hp-1* and *hp-1<sup>w</sup>* mutations into various genetic backgrounds for the purpose of improving fruit quality and nutritional value.

Fig. 1.

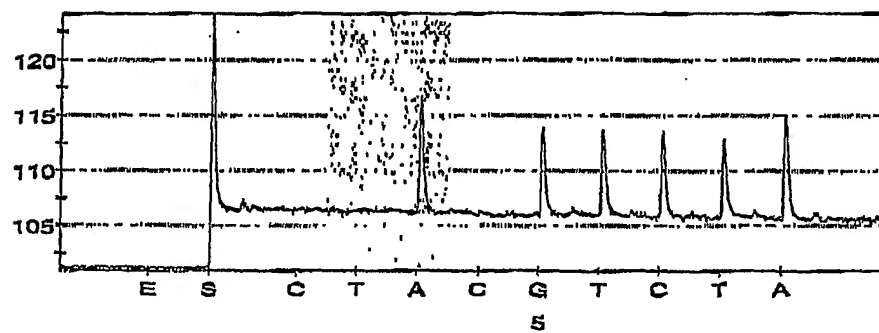
CTCATGAGAAGCAGAAGTCCGTCAGCATTTTCTAGACTGTCATTTCTACTTTAGCTGAGT  
TGCTGGGAATGAAATCTTCTCTTGACCCCTGCCTGGTTGCTGGAATAAAAATGTTTAAT  
TTGCATTGTTAACCCTGTTTTCCAGAGTTACCGGACTCAAAATTGAGCTACTGGGGGAAAC  
TTCTATTGCATCAACCATATCATACCTAGACA/TATGCTTTGCTTTCATTGGCTCAAG  
CTACGGAGATTACAGGTACTTTTAACTGTTGAGTGCATCTTGGTGCAATAAGTTGGTTT  
TTAGAGCTGCCFTATTGTATTTCCATACAGTAGCCTTTCATTCATTTGGAACATTGAGG  
TTTTAAATTTCAAGTGCCTATTTCTGGTGGTGCTTCATATTTACAGTTCCTACTAATATT  
TTTGAATTCACGTTTAGCTTGTAAGCTCAATCTCCAGCCTGACACCAA

**Fig. 2**

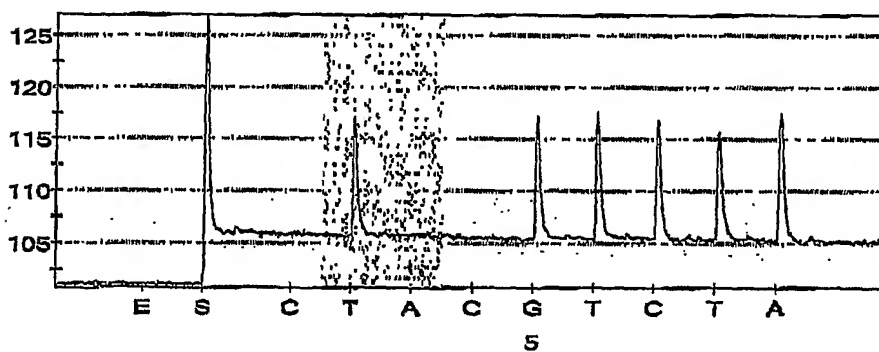


**Fig. 3**

a. *hp-1/hp-1*



b. *+/+*



c. *hp-1/+*

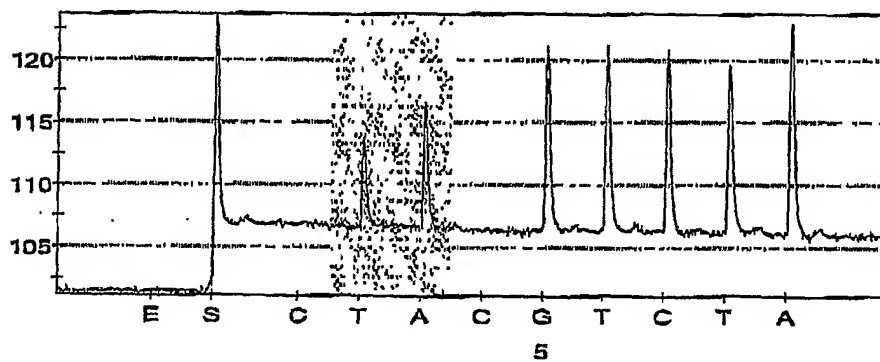




Fig. 4

a. hp-1 (Asn>Tyr)

Gg 285 LKREKQMDGTVTAKDMLKVELLGETSLAECLLYLDNGVVFVGSRUGDSQLVKLNEDSNEEG  
 Hs 285 LKREKQMDGTVTAKDMLKVELLGETSLAECLLYLDNGVVFVGSRUGDSQLVKLNEDSNEEG  
 Dm 285 LGTARTSG-VTVKD-KVPEQLGETSIPECLLYLDNGCLIGAREGDSQLVKLNSEKID-G  
 At\_DDB1B 282 LTHEK-----EAVTGLKIELLGETSLASSISYLDNVAVFVGSSGDSQLVKLNLPDARG  
 At\_DDB1A 282 LTHEK-----EAVTGLKIELLGETSLASTISYLDNVAVFVGSSGDSQLVKLNLPDARG  
 Le 282 LTHEK-----EAVTGLKIELLGETSLASTISYLDNVAVFVGSSGDSQLVKLNLPDARG  
 Os 283 LTHEK-----EAVTGLKIELLGETSLASSISYLDNGVVFVGSRUGDSQLVKLNLPDARG  
 Sp 293 ALFTD-----EVSFEERLGEBSIASCLIALPDNHMFVGSHEENSVLQLEPSITKN-

b. hp-1<sup>w</sup> (Glu>Lys)

Gg 817 MSCKLGKDDPNTYFIVGTAMVVPPEEAEKQGRIVVFHYS-DGKLQSPAEKEVKGAVYSVE  
 Hs 817 MSCKLGKDDPNTYFIVGTAMVVPPEEAEKQGRIVVFHYS-DGKLQSPAEKEVKGAVYSVE  
 Dm 818 MSACKLGDDPNTYFIVGTAMVVPPEEAEKQGRIVVFHYS-DGKLQSPAEKEVKGAVYSVE  
 At\_DDB1B 772 MSCKSFDDKNNVYCVGTAYVLPPEEAEKQGRILVPTVE-DGKLQSPAEKEVKGAVYSVE  
 At\_DDB1A 772 MSCKSFDDKNNVYCVGTAYVLPPEEAEKQGRILVPTVE-DGKLQSPAEKEVKGAVYSVE  
 Le 773 MSCKSFDDKNNVYCVGTAYVLPPEEAEKQGRILVPTVE-DGKLQSPAEKEVKGAVYSVE  
 Os 774 MSCKSFDDKNNVYCVGTAYVLPPEEAEKQGRILVPTVE-DGKLQSPAEKEVKGAVYSVE  
 Sp 762 LLENDDDR-----VVVGTETENFPEQDAPDSGRLEAVFEETSNNFEQAEHKVQGSVNLVL

Copy provided by USPTO from the IFW Image Database on 12/03/2004



1 MSVNVYVTAHQPTNVTHSCVGNFTGQELNLIIAKTRIEIHLITPQGLQPLMDVPYIGRIATLELERPHGETQDLIFI  
 81 ATERYKFCVLQWDTEASEVITRAMGVSDRIGRPTDNGOIGIDPDCLRLIGHLYDGLFKVIPPFDNKGQLKBAFNIRLEE  
 161 LQVLDIKFLYGCPRFTIVVLYQDNKDARHVKYEVSLKOKDFTEGEWAQNNLDNGASLLIPVPPPLCGVLLIGEETIVYC  
 241 SASAFKAIPIRPSITRAYGRVDADGSRVLLGDHNGLLHLIVITHEKVKVTEGLKIELLGETSIASTISYLDNAFVFIGSSY  
 321 GDSQLVKLNLPDPTKGSXVEVLEXYNLGPVDECVVDLERQGGQVWTCGAYKOGSLRIVRNGIGINEQASVELQGIK  
 401 GWSIRSATDDPYDTFLVVSFISETRVLAMNLEDELEETEIEGENSQVOTLFXCHDAVYMQLVQVTSNVRLVSSTSRDLK  
 481 NEMFAPVGYSVNVATANATQVLIATGGHLVYLIIGDGVLEVVKAKLDYDIISCLDINPIGENPNYSNIAAVGMWTDISV  
 561 RIYSLPDLNLIITKEQLGGEIIPRSVLMCSFEGISYLLCALGDGHLNFIWSMTGELTDRKKVSLGTQPTTLRTFSKDT  
 641 THVFAASDRPTVIYSENKLLYSNVNLKEVSHMCPFNVAAPDLSLATAKEGELFTIGTIDEIOKLHRSIPIGEHARRISH  
 721 QEQRTEALCSVKYTOQSNADDPHMHFVRLDDQTEFTISTYPLDQFEXGCSILSCSFSDDSNVYYCIGTAYVMPEENEP  
 801 KGRILNFIVEDGKLQLIAKETKGAAYYSINAFNGKLLAANQKIQLYKNASREDGGSRELQTECCGHHGILLALYVQTRGD  
 881 FIVVGDLMKSISILLIFKHBEGAIEERARDYNNANMSEAVEILLDDIYILGAENNFMLFTVRKNSKCATDEERSRLEVVGCVH  
 961 LGEFVNRFRHGSILVMRLPDESQVQIPTVIFGTVNGVIGVIAASLPDQXLFLEKIQTNLRKVTGKVGGLSHHQWRSFYNEK  
 1041 KTVDAKNFLDGDLEIESFLDLSRNRMEISKAMSVFVEELMKRVEELTRLH